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Protection of Rats Against Dental Caries by Passive Immunization With Hen-egg-yolk Antibody (IgY)

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Hen-egg-yolk antibody (IgY) was prepared against *Streptococcus mutans* MT8148 serotype c that was cultivated in medium containing sucrose, and it was used in passive caries-immunity studies. Specific pathogen-free rats infected with *S. mutans* MT8148 (c) and fed with a cariogenic diet containing more than 2% immune yolk powder developed significantly lower caries scores than did the ones infected with the same strain and fed with a diet containing only control yolk powder obtained from non-immunized hens. Similar results were obtained in an experiment with rats infected with *S. mutans* JC-2 (c) strain. Rats provided a diet supplemented with 0.5% immune water-soluble protein fraction containing *S. mutans*-specific IgY and challenged with *S. mutans* MT8148 exhibited significantly fewer caries lesions, compared with control rats on the normal diet.

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Introduction.

Streptococcus mutans serotype c is thought to be the principal causative bacterium of dental caries in humans (Loesche *et al.*, 1975; Bratthall and Köhler, 1976; Loesche and Straffon, 1979). Several studies have been made regarding preventive measures for protecting the host from this disease. There are many reports suggesting the possibility of preventing dental caries by vaccination (active immunization) using as antigens mutants streptococci whole cells or one of its characteristic cariogenic factors (McGhee *et al.*, 1976; Smith *et al.*, 1978, 1979; Michalek *et al.*, 1978, 1983; Lehner *et al.*, 1981). Many researchers have also reported the effectiveness of passive immunization against caries, in which specific antibodies against mutants streptococci are administered orally (McGhee *et al.*, 1976; Lehner *et al.*, 1978, 1985; Ma *et al.*, 1987; Michalek *et al.*, 1987). Recently, passive immunization has gained much attention, as compared with active immunization, because of the possible side-effects caused by mutants streptococcal vaccine antigens (Van de Rijn *et al.*, 1976; Hughes *et al.*, 1980; Stinson and Bergey, 1982).

It is known that the hen transfers serum IgG to the egg yolk and that this antibody gives immunity to its offspring (Patterson *et al.*, 1962). The antibodies present in egg yolk have been termed IgY (Hatta *et al.*, 1990). Thus, it is possible to obtain pathogen-specific IgY antibody from eggs laid by hens immunized against antigens (Bartz *et al.*, 1980; Shimizu *et al.*, 1988). Since poultry farming is carried out on a large scale globally, eggs may be a suitable source of antibody for passive immunization, which requires large amounts of antibodies.

In the present study, the preparation of egg-yolk antibody (IgY) against *S. mutans* serotype c and its effect on dental caries after passive immunization of rats were investigated.

Materials and methods.

Preparation of antigen.—The *S. mutans* MT8148 (c) strain was used as an antigen. The organism was cultured for 48 h in brain-heart infusion broth (Difco Laboratories, Detroit, MI) containing 5% (w/v) sucrose at 37°C in aerobic conditions. The micro-organisms were treated with 0.5% formalin for 24 h and were collected by centrifugation (10,000 g, 15 min). The pellet was washed three times with sterile saline containing 0.5% formalin, re-suspended in sterile saline, and homogenized by a homomixer (Phycostron®, Niti-On-Irika, Chiba, Japan). These samples were frozen until use after their concentrations had been adjusted to 1×10^9 equivalent colony-forming units/mL.

Immunization.—The 50 hens (150 days old) were immunized with 2 mL of the antigen (containing 1×10^9 CFU killed *S. mutans*) by intramuscular injection into both legs (Shimizu *et al.*, 1988). After initial immunization, the immunization was repeated once a week for four weeks (as indicated by arrows in Fig. 2). Immunization was repeated when the antibody titer decreased. The eggs were collected daily between four and ten weeks after initial immunization and stored at 5°C prior to antibody purification. Blood was also collected from the vein below the wing and used for analysis of antibody activity.

Partial purification of yolk antibody (IgY).—For the separation of IgY antibodies, an average of 2000 eggs was pooled from 50 immunized or non-immunized hens. Details of IgY purification have been extensively described elsewhere (Hatta *et al.*, 1988). Briefly, one part of the yolk was separated (High separator®, Suzusa Gohkin Mfg. Co., Ltd., Tokyo, Japan) from the eggs of immunized hens and mixed well with nine parts of 0.1% (w/v) λ -carrageenan (FMC Co., Philadelphia, PA) aqueous solution in order to remove yolk lipoprotein (Hatta *et al.*, 1990). After the mixture was allowed to stand for 30 min, the aggregated lipoproteins of the yolk were separated by centrifugation (10,000 g, ten min). The supernatant was filtered through No. 2 filter paper (Advantec Toyo, Tokyo, Japan). Disodium hydrogen phosphate (10 mmol/L) was added to the filtrate (water-soluble protein fraction; WSF), and the WSF was adjusted to pH 8.0 with 3 mol/L NaOH. This solution was lyophilized to obtain the immunized WSF powder used in the rat caries test.

For the preparation of immunized yolk powder, egg yolk separated from eggs of immunized hens (laid between four and ten weeks after initial immunization) was homogenized (T.K. Homomixer, Tokushu-Kika-Kogyo, Osaka, Japan) and spray-dried. Both control yolk powder and WSF powder were prepared from the eggs of non-immunized hens in the same manner as mentioned above.

The total protein amount in yolk powder and WSF powder was assayed by a modified Lowry method (Markwell *et al.*, 1978). To determine the relative amounts of IgY in WSF and yolk powder preparations, a single radial immunodiffusion (SRID) method was employed by the use of SRID plates containing 5% (v/v) anti-serum against chicken serum IgG (Zymed

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Summary of Experimental Protocol

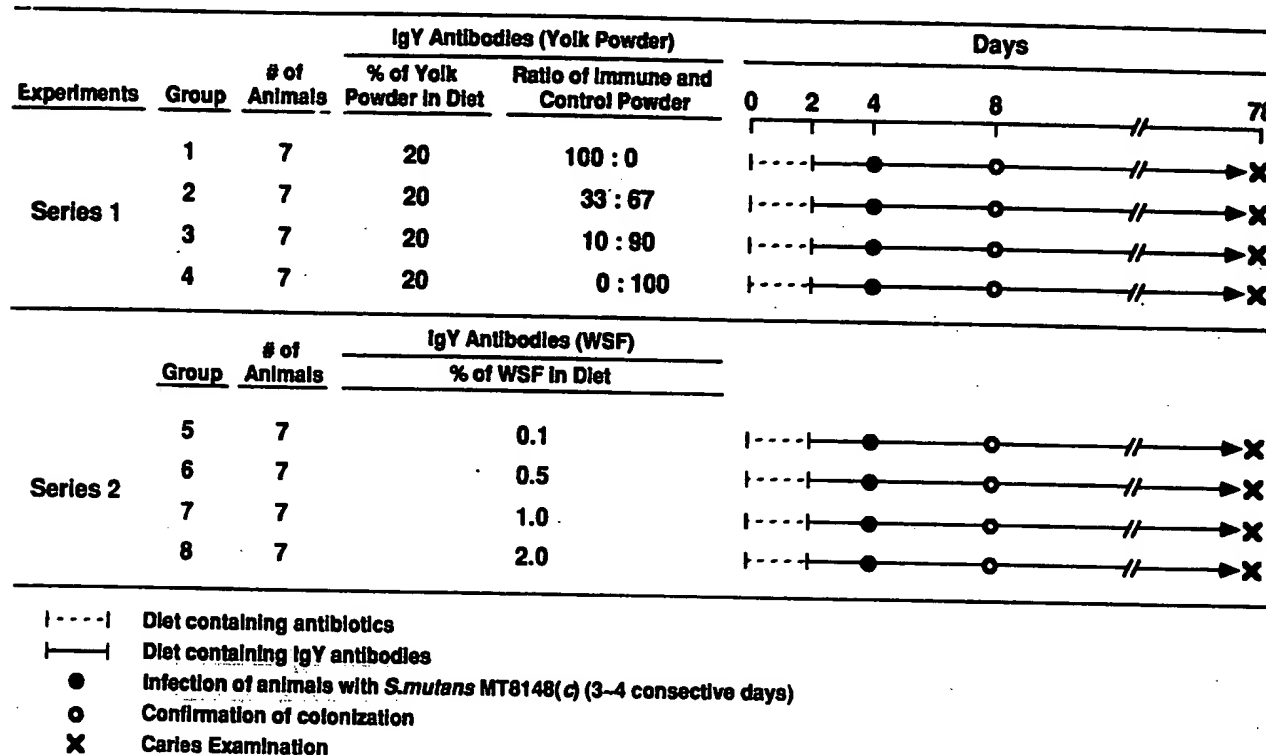


Fig. 1—Summary of experimental protocol. In experimental series 1, rats were fed with diet M2000 containing yolk powder prepared from immunized and/or normal hens. Further, rats were infected with *S. mutans* MT8148 or JC-2. In experimental series 2, rats were fed with diet 2000 containing IgY WSF preparations and infected with *S. mutans* MT8148.

Laboratories Inc., San Francisco, CA) (Hatta *et al.*, 1990). Purified chicken serum IgG was used as a standard. The WSF preparation contained approximately 400 mg of IgY in 3500 mg of yolk protein obtained from 100 g of egg yolk (Table 1). The crude yolk powder fraction consisted of approximately 2% IgY. Specificity of IgY antibody was examined by the Ouchterlony test with rabbit antiserum against chicken serum IgG and mouse IgG. Specific binding was only seen with anti-chicken serum IgG.

Determination of antibody levels.—(1) *Enzyme-linked immunosorbent assay (ELISA).*—The time-course of antibody levels in egg yolk against *S. mutans* (c) was evaluated by the ELISA method of Cost *et al.* (1985). *S. mutans* antigen was suspended in 0.1 mol/L carbonate buffer (pH 9.6). This antigen suspension (A660 nm = 1.0) at 100 μ L/well was used for coating an ELISA plate. Ovalbumin (1 mg/100 μ L) was used for blocking. Each well was washed three times with 200 μ L of phosphate-buffered saline (PBS) containing 0.05% Tween 20 (PBS-Tween). Egg yolk and chicken serum were obtained from different time points and always diluted 1000-fold with PBS-Tween; 100 μ L of each *per* well in triplicate was reacted with the antigen for two h at 37°C. After each well was washed again with PBS-Tween (as mentioned above), alkaline-phosphatase-conjugated rabbit IgG anti-chicken IgG (Zymed Laboratories, Inc.) diluted 2000-fold with PBS-Tween (100 μ L) was added to each well, and the plate was incubated for two h at 37°C. Each well was washed with PBS-Tween, and 100 μ L of the substrate solution (*p*-nitrophenyl phosphate, 1 mg/mL diethanolamine buffer, pH 9.8, Sigma, St. Louis, MO) was added. Enzyme reaction (30 min at room temperature)

was stopped by adding 5 mol/L NaOH (50 μ L/well), and the color developed was read at 405 nm with a plate reader (Model 2550, Bio-rad, Richmond, CA). As a control, serum samples obtained from non-immunized hens were used. In some experiments, purified IgG from serum of immunized hens was used as a standard.

(2) *Agglutination titer.*—The agglutination titer of the yolk powder and WSF powder used in the rat caries experiment was determined by a microtiter-plate (Sanko-Jun-Yaku, Toyo, Japan) technique. Each sample was dissolved in 10 mmol/L PBS, pH 7.2 (50 mg powder/mL). After each mixture was centrifuged (10,000 g, 15 min), 50 μ L of each supernatant was

TABLE 1
PREPARATION OF ANTI-*S. mutans* MT8148 (c) EGG-YOLK ANTIBODIES

Sample	Protein	% IgY	<i>S. mutans</i> -specific IgY	
			Agglutination Titer	ELISA Value (A 405 nm)
Immunized Yolk Powder	30.6%	1.6%	32	0.6
Control Yolk Powder	30.9%	1.2%	<2	0
Immunized WSF Powder	71.3%	10.4%	254	0.9
Control WSF Powder	74.8%	10.8%	<2	0

WSF: Water-soluble protein fraction.
IgY: Immunoglobulin yolk.

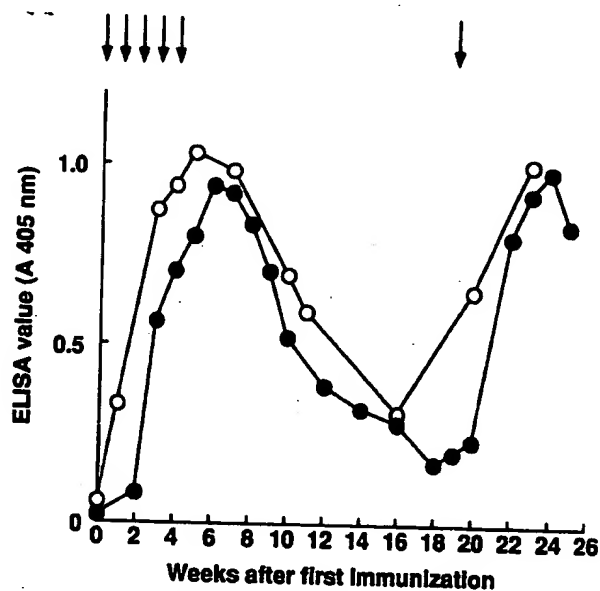


Fig. 2—Egg-yolk and serum antibody values against *Streptococcus mutans*. Closed (●) and open (○) circles indicate antibody levels in egg yolk and serum, respectively, of hens immunized with *S. mutans* MT8148. Arrows indicate the immunization schedule. Intramuscular immunization was performed once a week for four weeks after initial immunization. Boosting was also done at 19 weeks. The data in this Fig. are representative of results obtained from ten immunized hens.

mixed with the *S. mutans* (c) whole-cell antigen (A660 nm = 1.0). After the plate remained for two h at 37°C, it was further incubated for 12 h at 5°C, and the observed agglutination titer was recorded. As standard and control, serum or purified IgG obtained from hens immunized with *S. mutans* were used in this study.

Experimental rat caries model.—In order to test anti-cariogenic activity of IgY antibodies *in vivo*, the experimental rat caries model was used. Specific pathogen-free Sprague-Dawley rats (20 days of age) (Japan Clea Lab., Tokyo) were treated with ampicillin, carbenicillin, and chloramphenicol (Michalek and McGhee, 1977) for three days to eliminate the microbial flora. To this end, rat chow (Oriental Yeast Co., Ltd., Tokyo) was pulverized and mixed with 1 g of each individual antibiotic per 1 kg of diet. This treatment reduced the number of oral micro-organisms when oral swabs were obtained from individual rats and examined on plates containing BHI and mitis-salivarius agar (Difco Laboratories, Detroit, MI). *S. mutans* was not detected in the oral cavity. Rats were randomly separated into several experimental groups, and each group contained seven rats (Fig. 1). In a series of experiments (series 1), all rats were fed modified diet 2000 (M2000), in which 56% sucrose in diet 2000 (Keyes and Jordan, 1964) was replaced by 36% sucrose and 20% yolk powder, which contained immunized and/or control yolk powder. The rats were then infected with streptomycin-resistant (1 mg/mL) strains of *S. mutans* MT8148 (c) or *S. mutans* JC-2 (c) by pipette (50 μ L of 1×10^{11} CFU/mL) at 24 days of age (Fig. 1). Four groups of rats were fed diets containing four different ratios of anti-*S. mutans* MT8148 (c) immunized yolk and control yolk powder, as follows: 100/0 for group A; 33/67 for group B; 10/90 for group C; and 0/100 for group D, respectively. Oral swabs were taken to confirm colonization of the inoculum. The rats were killed at 78 days of age, and the mandibular caries scores were measured by the method of Keyes (1958).

In a separate series of experiments, WSF powder preparation

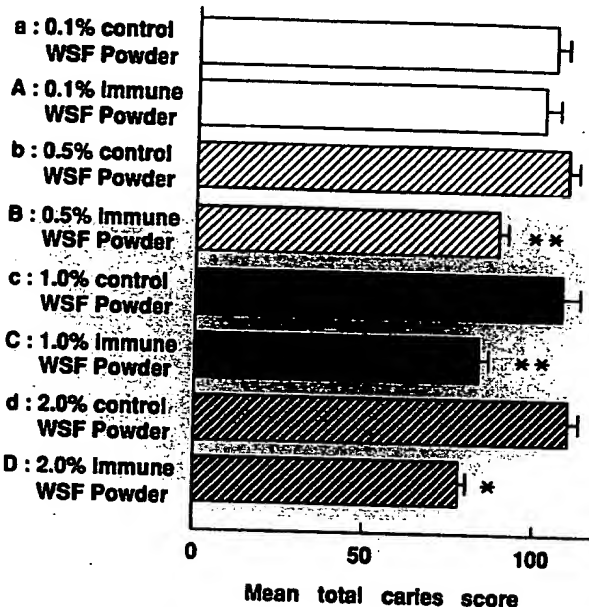


Fig. 3—Mean caries scores in mandibular molars of specific pathogen-free rats infected with *S. mutans* MT8148 (c) and fed with diet 2000 containing WSF for 58 days. Significance of differences: ** $p < 0.05$, * $p < 0.01$.

containing higher amounts of IgY was used in order to examine anti-cariogenic activity *in vivo*. In this experiment, rats were fed diet 2000 containing 56% sucrose. Four different amounts of WSF powder obtained from *S. mutans*-immunized and non-immunized egg yolk were added to diet 2000. Groups A, B, C, and D contained 0.1, 0.5, 1.0, and 2.0% of anti-*S. mutans* immunized WSF powder in diet 2000, respectively. As controls, identical amounts of WSF powder prepared from normal egg yolks were added to diet 2000, and these groups were indicated as a, b, c, and d. All animals were infected with *S. mutans* MT8148 (c).

Statistical analysis.—The caries scores were statistically analyzed by computing means and standard errors of the mean. Differences between means of the experimental and control groups were evaluated by Student's *t* test. The *p* value was always established by comparison of the means obtained from individual experimental and control groups only.

Results.

Changes of antibody levels.—Changes of antibody levels in both egg-yolk and chicken serum against *S. mutans* antigen were investigated by ELISA for 25 wk after the initial immunization (Fig. 2). When immunization was made once a week for five wk, the IgY antibody level in egg yolk increased from the third week, reaching its peak in the sixth week. On the other hand, the antibody level in the serum began to increase from the first week, reaching its peak in the fifth week. Subsequently, the antibody level decreased gradually, but when an additional immunization was conducted in the 19th week, the maximum level of the antibody was recovered in each hen.

Purity of yolk antibody and agglutination titer.—The agglutination titers against *S. mutans* (c) antigen in the immune yolk powder and the immune WSF powder were 32 and 256, respectively (Table 1), whereas the agglutination titers of both control yolk powder and WSF powder were below 2.

The content of antibody in the WSF powder was about ten times higher than that in the yolk powder.

TABLE 2
MEAN CARIES SCORES IN MANDIBULAR MOLARS OF SPECIFIC PATHOGEN-FREE RATS INFECTED WITH *S. mutans* MT8148 (c) AND FED WITH DIET M2000 FOR 58 DAYS†

Groups (Ratio of Immune/Control Yolk Powder in Diet)‡	Mean Caries Score (\pm SE)§			
	Sulcal	Buccal	Approximal	Total
A (100/0)	40.1 \pm 2.5*	2.1 \pm 0.3*	0.5 \pm 0.3*	42.8 \pm 2.8*
B (33/67)	64.1 \pm 3.7*	5.6 \pm 0.4**	1.4 \pm 0.6*	71.1 \pm 4.1*
C (10/90)	71.1 \pm 5.0*	7.3 \pm 1.2	3.0 \pm 0.8	81.4 \pm 6.5*
D (0/100)	90.4 \pm 1.1	9.6 \pm 1.1	4.9 \pm 0.8	104.9 \pm 1.9

†56% sucrose in diet 2000 was replaced by 36% sucrose and 20% egg-yolk powder containing different ratios of immune and control powders.

‡Different ratios of egg-yolk powder prepared from immunized and normal hens were added to diet M2000. For example, diet M2000 used in group B contained a ratio of 33 to 67 of immune and control powder, respectively, in the 20% egg-yolk powder. Each group contained seven rats. The average weight of the rats was 234.1 \pm 3.2 g, and the weights of rats in the four different groups were very similar.

§Statistical analyses (*t* tests) were carried out between group D and the other groups.

*Significance of difference, $p < 0.01$.

**Significance of difference, $p < 0.05$.

Effect of egg-yolk IgY antibody on rat caries—(1) Effect of immune yolk powder on rat caries.—The mean mandibular caries scores of rats infected with *S. mutans* MT8148 (c) strain were decreased by increasing the ratio of the immune yolk powder (0 to 100%) over control powder in the diet (Table 2). As low as one part of immune powder to nine parts of control powder in the 20% yolk protein contained in the M2000 diet reduced caries development in the sulcal surfaces (group C) when compared with control group D, which was fed with diet containing the control yolk powder only. Interestingly, the most significant reduction of caries development was achieved when immune yolk protein constituted 20% of the diet. The average body weights of the rats in the different groups were not significantly different from each other. Similar results were obtained in the experiment on the rats infected with *S. mutans* JC-2 (c) strain (Table 3). In this experiment, rats received diet M2000 containing different amounts of IgY (anti-*S. mutans* MT8148) and were infected with *S. mutans* JC-2 (c). Feeding of rats with this IgY antibody also reduced the development of caries induced by *S. mutans* JC-2. These results demonstrated that yolk protein containing *S. mutans* serotype c-specific IgY antibody in M2000 reduced the development of dental caries.

(2) Effect of immune WSF powder on rat caries.—Statistically significant differences in total caries scores were observed in the groups fed the diets containing 0.5, 1.0, and 2.0% of immune WSF powder, compared with the group fed control WSF powder (Fig. 3; $p < 0.05$ for 0.5% and 1.0%, $p < 0.01$ for 2.0%). When the effect of diet 2000 containing immune WSF powder on smooth surface and sulcal caries was examined, 0.5% immune WSF significantly inhibited development of approximal rather than sulcal caries (data not shown).

Diet 2000 containing 2% immune WSF inhibited both smooth and sulcal caries development. On the other hand, diet consisting of control WSF did not provide any protection (Fig. 3).

Discussion.

Passive immunization has been attempted as a method for prevention of dental caries. The present study was conducted to determine the effectiveness of egg-yolk antibody (IgY) obtained from hens immunized against *S. mutans*. The yolk antibody against *S. mutans* (c) in this study showed a remarkable preventive effect against rat caries caused by *S. mutans* serotype-c infection (Tables 2, 3; Fig. 3).

It has been known that serum IgG of the hen is transferred to its egg yolk and provides its offspring with acquired immunity (Patterson *et al.*, 1962), and this antibody in egg yolk has been termed IgY (Hatta *et al.*, 1990). Therefore, we immunized hens with *S. mutans* serotype (c), cultured in medium containing 5% (w/v) sucrose, as an antigen. The antibody levels against *S. mutans* antigen in the egg yolk were raised one or two weeks after that in the serum (Fig. 2). To this end, although both primary and secondary immunization induced a high titer of antigen-specific IgY antibody in the egg yolk, induction of *S. mutans*-specific antibody was noted two to three weeks later, when compared with serum antibody. To achieve the highest titer of *S. mutans*-specific antibody in egg yolk, the secondary immunization required only one boosting, while at least five administrations were required for the primary immunization. This finding indicated that similar immunization schedules, as with other experimental animals (e.g., mice and rabbits), were required for the induction of antigen-specific antibody in hen-egg yolk.

TABLE 3
MEAN CARIES SCORES IN MANDIBULAR MOLARS OF SPECIFIC PATHOGEN-FREE RATS INFECTED WITH *S. mutans* JC-2 (c) AND FED WITH DIET M2000 FOR 58 DAYS†

Groups (Ratio of Immune/Control Yolk Powder in Diet)‡	Mean Caries Score (\pm SE)§			
	Sulcal	Buccal	Approximal	Total
A (100/0)	54.8 \pm 1.4*	1.7 \pm 0.4*	0.5 \pm 0.5*	57.0 \pm 1.5*
B (33/67)	67.2 \pm 1.2*	5.2 \pm 0.5**	1.2 \pm 0.6**	73.6 \pm 1.6*
C (10/90)	73.6 \pm 3.2*	6.8 \pm 1.4	2.4 \pm 0.7	82.8 \pm 4.3*
D (0/100)	94.7 \pm 2.9	8.7 \pm 0.9	3.5 \pm 0.6	106.8 \pm 3.2

†See Table 2, footnote †.

‡See Table 2, footnote ‡. The average weight of the rats was 234.3 \pm 3.4 g, and the weights of the rats in the four different groups were very similar.

§See Table 2, footnote §.

*See Table 2, footnote *.

**See Table 2, footnote **.

It has been demonstrated that adsorption of *S. mutans* MT8148 (c) to saliva-coated hydroxyapatite is remarkably inhibited by pre-treatment of *S. mutans* (c) with the purified yolk antibody, IgY (manuscript in preparation). We suggest that part of the rat caries reduction by the IgY antibody was due to inhibition of *S. mutans* (c) cell attachment. However, no statistically significant differences were obtained in the number of *S. mutans* in the experimental groups, compared with the control.

It has been reported that only the immunoglobulin G class of antibody exists in egg yolk (Heller, 1975). Immunological identity of the yolk antibody with the chicken serum IgG has also been demonstrated (Hatta et al., 1990). Therefore, it is considered that the caries protection in the rat that was observed in this study resulted from the IgY of antibody in the egg yolk obtained from immunized hens.

Recently, researchers have reported the possibility of preventing caries by passive immunization using mouse monoclonal IgG against *S. mutans* cell-surface protein antigen I/II (Lehner et al., 1985) and immunized bovine whey IgG1 against *mutans streptococci* whole cells (Michalek et al., 1987). Our study provides additional evidence that *S. mutans*-specific IgY antibody induced in the yolks of hens' eggs can be used for passive immunization to prevent development of dental caries in rats.

The content of antibody in the egg yolk (ca. 100–150 mg/egg) is remarkably higher than that in mammalian serum or milk. Moreover, eggs containing antibody are consumed as food. Considering their safety, it may be no problem to use eggs as a source of antibody for the prevention of dental caries by passive immunization. Passive immunization (orally administered yolk antibody against *S. mutans*) may provide a new approach to the prevention of dental caries.

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